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**Cytospin smears in veterinary cytology: a quick, simple and affordable manual
method**

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Running title: In-house cytospin manual method

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26

27 **Abstract**

28 **Background:** Smears prepared in cytocentrifuges, the so-called cytospins, are widely
29 used in human cytology. In the veterinary field, the high cost of the equipment has
30 hampered a widespread use of cytospins in veterinary clinics. Nevertheless, cytospins
31 are important for evaluating fluids, especially those with low cellular content, such as
32 cerebrospinal fluid (CSF) or bronchoalveolar lavage fluid (BALF).

33 **Objectives:** The aim of this study was to devise and test the use of a low-cost, in-house
34 centrifuge to obtain cytospin preparations.

35 **Methods:** Twenty-two fluid samples (including CSF and BALF) were collected from
36 dogs and cats. These were processed in a conventional cytocentrifuge and in an in-
37 house, manual centrifuge (salad spinner). The cytospins obtained by the two methods
38 were compared by scoring cellularity, number of cells per field, hemodilution, cell
39 preservation and amount of ruptured cells. Additionally, cell number and size was
40 compared by morphometry. Differences between the cytospin methods were statistically
41 assessed.

42 **Results:** The morphology and cellular detail of cytospins produced by both methods
43 were identical. An almost perfect agreement was observed for cellularity, number of
44 cells per high power field, hemodilution and cell preservation ($\kappa \geq 0.85$) and a
45 moderate agreement for the amount of ruptured cells. Cell recovery was comparable,
46 even in low cellular samples, such as CSF and BALF.

47 **Conclusions:** The manual centrifuge produced cytospins similar to those of the
48 conventional cytocentrifuge. Considering the low cost and portability, this new method
49 should be particularly useful for cytological diagnosis in small clinics, developing
50 countries and in field studies.

Introduction

The study of fluid samples [i.e., effusions, cerebrospinal fluid (CSF), bronchoalveolar lavage fluid (BALF) or urine] is relevant for the diagnosis, treatment and follow-up of inflammatory and neoplastic conditions both in human and veterinary medicine. Except for CSF, most veterinary practitioners collect such samples on a weekly basis.¹ In low cellular fluids, it is often mandatory to make concentrated slides to accurately evaluate cellular morphology. Smears prepared in cytocentrifuges, the so-called cytopsin smears or cytopsins, are widely used in human cytology to concentrate cells.² In cytopsins, cells are automatically monolayered in a small area of the slide, enabling a fast and reproducible observation. Nowadays, cytopsin preparations are considered the best method for cell concentration in veterinary medicine,^{3,4} but cytopsins are much less used in the veterinary than in medical field, except for the analysis of BALF and CSF.^{5,6} Probably, this is due to the high price of cytocentrifuges that hampers the wide use of cytopsins in veterinary clinics.⁷ In many occasions, fluid samples are often sent by mail to laboratories that later on make cytopsins, but this may compromise the diagnose due to *in vitro* cell degradation. Cells may be affected by aging changes occurring in as few as 24 hours, which can alter the cell pool in inflammatory samples⁸ and may even compromise the assessment of neoplastic criteria.⁹ This is especially true for CSF and BALF, which should be processed, ideally, within 1 and 2 hours after the sampling procedure, respectively.^{6,10} In this vein, an affordable, in-house and quick method for obtaining cytopsins from fluid samples would be of value to veterinary practitioners.

The aim of this study was to devise a new and low-cost technique to generate cytopsin preparations. The method was aimed to give good cellularity and morphology, comparable with samples processed in cytocentrifuges. In order to be used by veterinary

practitioners, an alternative procedure should be, at least, equally fast, simple and consistent, but more affordable.

Materials and methods

Cytological specimens

Twenty-two fluid samples from dogs (20 cases) and cats (two cases) received for diagnostic evaluation were studied. These comprised 11 cavitory effusions (six thoracic, three peritoneal and two pericardial), eight CSF, two BALF, and one urine. An equal amount of liquid (200µl) was processed in a conventional cytocentrifuge (StatSpin Cytofuge 2® Inc, Norwood, Massachusetts, USA) and by an alternative manual method described below. It is opportune to mention that, in all cases, processing occurred within 30 minutes (CSF) or up one hour after sample collection.

Cytospins produced by the conventional method

Samples were centrifuged for six minutes in StatSpin Cytofuge 2® at 140g (1,600 RPM) in all cases except for CSF [eight minutes at 40g (850 RPM)], following manufacturer's recommendations. The obtained cytospins served as reference for the alternative manual procedure. In all cases, reusable cell concentrators (VWR cat: 720-1972), with disposable filters with a central hole of 7.25 mm (VWR cat 720-1973) fixed with clips (StatSpin® cat: FFCL) were used.

Cytospins produced by the alternative manual method

A commercial salad spinner (26 cm diameter, Zyliss® cat: 15201, Diethelm Keller brands, Zurich, Switzerland) was used (Fig. 1). The handle was pulled continuously to half distance of the string (90 to 100 pulls per minute controlled by a wall watch). In

average, the basket rotated at 127g for five minutes (1,150 RPM) as measured by a digital tachometer (DT-2234C, Rinch Industrial, China, accuracy ± 1 RPM). The same time and rotation was used for all fluid samples (including CSF). Styrofoam cushions of 5 x 3 x 2 cm were fitted to the basket with rubber bands (Fig. 2), which also held the cell concentrators (opposite sides of the basket were selected, in order to keep it balanced). For sample processing, reusable cell concentrators, disposable filters, and metallic clips similar to those of the conventional method were applied. To maximize cell recovery, samples were spun within few seconds after filling the chambers. All slides were stained with a commercial Romanowsky-type stain (Hemacolor, Merck, Darmstad, Germany) and mounted with mounting media (Coverquick 2000, VWR Chemicals, Fontenay-sous-bois, France).

Qualitative and quantitative comparison between samples

For the comparing the two methods, slides were coded and examined blindly to the method by an experienced board-certified cytopathologist (MC). Samples were assessed by scoring on a 1-3 scale the following parameters: cellularity (1 = low, 2 = moderate, 3 = high); cell preservation (1 = poor, 2 = moderate, 3 = good); amount of ruptured cells per 60x-high power field (HPF) (1 = less than 10%, 2 = between 10 and 50%, 3 = more than 50%). A semi-quantitative evaluation of hemodilution (1 = less than 10 red blood cells (RBC), 2 = between 10 and 100 RBC, 3 = more than 100 RBC per HPF) and of the number of nucleated cells per HPF (1 = less than 10, 2 = between 10 and 100, 3 = more than 100 per HPF) was also performed.

In order to further compare the methods, cell number and size was assessed by morphometry. Photos from each quadrant and central part of the circular area were taken at 100x-oil immersion field (OIF) and the average cell diameter assessed with the

ImageJ software 1.47v (<http://imagej.nih.gov/ij>). Since neoplastic cells have a marked anisocytosis, only the size of erythrocytes and neutrophils was considered (25 cells per case, on average). Additionally, the number of all nucleated cells in these OIF was assessed.

For the statistical analysis, the software SPSS18 (IBM, Armonk, USA) was used. Differences between scores were assessed using the Wilcoxon signed-rank test, with a Bonferroni correction (statistical significance set at $p < 0.05$). The agreement between the two cytopsin methods was assessed with kappa statistics. For interpreting the strength of agreement, the following standards were considered: ≤ 0.40 = poor, $0.41-0.60$ = moderate, $0.61-0.80$ = good and $0.81-1$ = almost perfect.¹¹ For the differences in the cell diameters and number of cells per OIF, the Mann-Whitney U test was applied.

Results

Results of the cytological evaluation are depicted in Table 1. Processing samples in both centrifuges resulted in good quality cytopsins, with cells distributed over the circular area which roughly corresponds to the area covered by the 4x objective. The manual method was straightforward to use: inserting and removing the cell concentrators was easy, and pulling the handle for five minutes was manageable. In general, cytopsins obtained by the two methods were highly comparable (Fig. 3).

The size of erythrocytes and neutrophils was similar in both methods, being 6.2 ± 1.0 μm and 5.6 ± 0.7 μm with manual and conventional cytopsins, respectively for erythrocytes, and 11.2 ± 2.1 μm and 12.2 ± 2.1 μm with manual and conventional cytopsins, respectively for neutrophils. The median of nucleated cells per OIF was 11 (range 0-22) and 13 (range 0-46) in manual and conventional cytopsins, respectively, without significant differences.

The semi-quantitative analysis also showed no significant differences between samples from both methods (Table 2, Fig. 4). An almost perfect agreement was observed for cellularity with a kappa of 0.85 [95% confidence interval (CI): 0.69-1.00]. Indeed, a kappa of 0.92 (CI: 0.85-1.00) was estimated for the number of cells per HPF. The hemodilution and cell preservation were similar with both methods [kappa = 0.93 (CI: 0.81-1.00) and 0.92 (CI: 0.84-1.00), respectively]. A moderate agreement existed for the amount of ruptured cells [kappa = 0.47 (CI: -0.29-1.00)].

Discussion

Cytospins produced by conventional and manual methods were compared using a qualitative and quantitative approach in order to assess if an in-house, low-cost centrifuge would produce cytopins suitable for cytological diagnosis.

Speed, simplicity and low-cost are paramount for a general use of any diagnostic method. The high cost of the equipment has hampered a wide use of cytopins in veterinary clinics,⁷ and this technique has been confined to large diagnostic laboratories. Similarly, the cost of equipment has limited the use of cytopins in human cytology in developing countries.¹² Overall, the manual method devised was simple, generated cytopins as quick as the standard cytocentrifuge, but costed 100 times less (Zyliss salad spinner costs about 34\$, whereas the current quote of Statspin Cytofuge 2 exceeds 3600\$). Considering that the cell concentrators and metallic clips are reusable (costing 70c and 21\$ per unit, respectively), the final cost of a cytospin resumes to the used filter and slide, which are fairly inexpensive (filter costs 30c per unit). Still, these costs may vary, because material from other brands can be used (e.g., Shandon Cytospin®) and the metallic clips can be even replaced by adapted paper binder clips (Fig. 5).

Fluids, especially of low cellularity, should be processed as fast as possible to avoid cellular changes. If a cytocentrifuge is not available, fluid can be sedimented in a conventional centrifuge and a smear can be made, after pouring off the supernatant and resuspending the cell pellet. However, this often leads to suboptimal results,^{3,6,13} since cells may be distorted while deposited and smeared onto a slide.² Moreover, smearing the cell button is impracticable in low cellular fluids, such as CSF or even BALF, in some instances. In such cases, gravity sedimentation chambers can be built with in-house material,^{3,14,15} but these require up to 60 minutes for cell sedimentation and also generate slides of lower quality compared to cytopsins.⁵ With the manual method presented herein, veterinary practitioners can easily perform a cytopsin for immediate diagnose, and this smear can be sent later on for a more detailed evaluation by the laboratory, without losing any morphological cellular feature or changing the cell differential, that is inevitable to a long storage of fluids.¹⁰ This may solve the classic dilemma of having a CSF for diagnostic evaluation when all laboratories are closed. Manual hand-powered centrifuges have been developed for molecular biology¹⁶ and hematology uses¹⁷ but this is the first time that they are applied for cytology. These centrifuges are not practical in large laboratories dealing with a high number of samples in a daily basis, but they are valuable in small clinics that occasionally need to process liquid samples. It is opportune to mention that the method developed herein can be also applied for other species in which cytopsins have proved their value, such as in the evaluation of BALF in horses¹³ or even in milk evaluation from dairy cows.¹⁸ Taking into account that the salad spinner is highly portable and hand-powered, their use would be particularly convenient under field study conditions. In conclusion, the manual method seems a valuable option to produce cytopsins, being well-suited for low cellular samples such as CSF and BALF. Considering their

simplicity, speed and low-cost, this method could be included in the toolbox of veterinarians devoted to cytological diagnosis in very small clinics, developing countries and under field conditions.

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Figure legends

Figure 1. The manual method uses an alternative centrifuge (A), which is hand-powered by pulling the handle (B).

Figure 2. With the manual method, up to 6 cell concentrators can be accommodated at each time using styrofoam cushions. After assembling the cell concentrator (with the slide, filter and metallic clip), it is fitted in the styrofoam cushion (arrow) using rubber bands (A) and the fluid inserted in the funnel (block arrow in B).

Figure 3. Cytospins produced by the manual and conventional cytopsin procedures.

Figure 4. A and B – Cytospins generated by conventional (A) and manual method (B).

Inflammatory pleural effusion from a dog. Neutrophils, macrophages and mesothelial cells in an hemodiluted background. C and D – Neoplastic pleural effusion from a dog with a history of mammary gland adenocarcinoma. Anysocytosis, anysokariosis, nuclear molding (arrowhead) and atypical mitotic figures (arrow) can be seen in conventional (C) and manual (D) cytopsin. E and F – Bronchoalveolar lavage fluid of a dog. Inflammatory cells, including eosinophils (arrows) appear along with ciliated and goblet cells in conventional (E) and manual (F) cytopsin. H and I – Cerebrospinal fluid of a moderate eosinophilic pleocytosis. Monocytoid cells, eosinophils and neutrophils (inset) are similar in conventional (H) and manual (I) cytopsin. Hemacolor; bar = 375 μm (I, H), 120 μm (A, B), 65 μm (inset of A and B), 25 μm (C, D, E, F, and inset of I and H).

Figure 5. Cell concentrators, filters and clips from other brands can be used in the manual method (A, Cytospin Shandon® and B, StatSpin Cytofuge 2® material). The metallic clips can be also replaced by adapted paper binder clips (C).